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Association of a Baculovirus-Encoded Protein with the Capsid Basal Region

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An open reading frame homologous to AcMNPV ORF9 (ORF1629) was characterized in the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus (OpMNPV). Sequence analysis indicated that the OpMNPV homolog (called ORF2) encoded a protein predicted to contain 545 amino acids with a molecular weight of 61 kDa. The first 80 amino acids did not have a counterpart in the AcMNPV homolog. The remainder of the ORF was poorly conserved with 29% amino acid identity overall with the AcMNPV ORF. However, the amino terminal 150 amino acids of AcMNPV ORF9 demonstrated about 45% amino acid sequence identity with OpMNPV ORF2 and conserved runs of proline residues were present in internal regions of both molecules. Transcriptional mapping indicated the ORF2 transcripts were initiated at a late promoter sequence, ATAAG, beginning about 24 hr p.i. These transcripts terminated near the 3′ end of the ORF2 reading frame. Antibodies were produced against a fusion protein derived from the bacterial gene encoding the maltose binding protein and most of the ORF2 sequence. These antibodies reacted with a protein of 69 kDa on Western blots and the protein was found to be associated with virions isolated from both polyhedra and budded virus. The OpMNPV ORF2 antiserum also reacted with the AcMNPV ORF9 gene product. Immunoelectron microscopic analyses indicated that ORF2 was associated with the ends of the capsids which contain the basal structure. This end appears to be oriented away from both the virogenic stroma and membranes involved in intranuclear envelopment. In addition, as virions bud from the nucleus into the cytoplasm, this end also appears to be oriented away from the nuclear membrane.

INTRODUCTION

Nuclear polyhedrosis viruses (NPV) produce two virion phenotypes, the budded virus (BV), which are involved in spreading the infection from cell to cell, and the occlusion-derived virus (ODV), which spread the infection between insects. BV are formed from single nucleocapsids that originate in the nuclei of infected cells. They then bud through the nuclear membrane, picking up a membrane which is subsequently lost in the cytoplasm and acquire a new membrane as they bud through the plasma membrane (Granados and Lawler, 1981). ODV, on the other hand, are enveloped in the nucleus and have multiple capsids per envelope in multinucleocapsid viral strains (MNPVs). Groups of enveloped virions are then occluded in a crystalline matrix to form polyhedra. Based on electron microscopy, the ends of baculovirus virions appear to have a different structural organization and these structures are called the apical cap and the basal structure (Fraser, 1986). The apical cap structure appears to be oriented toward the virogenic stroma and is thought to mediate the incorporation of nucleoprotein into preassembled capsid sheaths (Fraser, 1986). In virions destined to become ODV, the apical cap also appears to be involved in envelope acquisition. In addition, in virions destined to become BV the apical cap is thought to be involved in interacting with both the nuclear and plasma membranes during the budding process (Fraser, 1986). To date, no proteins have been identified that specifically associate with either the apical or basal end of the virion.

A number of baculovirus structural proteins have now been characterized that are present in both BV and ODV forms. The *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) encodes a late gene called *orf9* (Ayres *et al.,* 1994) or *orf1629* (Pham and Sivasubramanian, 1992; Pham and Sivasubramanian, 1993; Possee *et al.,* 1991) that is located adjacent to and in the opposite orientation to the polyhedrin gene. This orf is predicted to encode a protein of 543 amino acids with a molecular weight of 61 kDa. Insertional mutagenesis at the carboxyl terminal region indicated that ORF9 is essential for AcMNPV viability (Possee *et al.,* 1991) and this observation was exploited to develop a protocol for the selection of AcMNPV recombinants at high frequency (Kitts and Possee, 1993). Subsequently, Vialard and Richardson (1993) characterized expression of ORF9 protein and found that both unphosphorylated and phosphorylated forms with apparent molecular masses of 78 and 83 kDa, respectively, were present. Furthermore, they demonstrated by Western blot analysis that ORF9 was associated with both budded and occluded virions. Examination by immunoelectron microscopy indicated that the protein was localized to end structures of mature nucleocapsids but they did not determine whether it was specifically

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. U75930.

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associated with the basal structure or apical cap region. Although ORF9 appears to be an essential component of a highly conserved virion structure, it is poorly conserved. AcMNPV ORF9 has only 29% amino acid sequence identity with its homolog from *Heliocoverpa zea* SNPV (HzSNPV) (Cowan *et al.,* 1994).

As part of a program to identify and characterize baculovirus gene products associated with the virions of the *Orgyia pseudotsugata* MNPV (OpMNPV), we characterized the expression and location of OpMNPV *orf2* which is homologous to AcMNPV *orf9.* In this report, we describe the sequence and transcriptional mapping and temporal expression of this gene, as well as the localization of the *orf2* gene product in OpMNPV-infected *Lymantria dispar* cells.

MATERIALS AND METHODS

Insect cell lines and virus

A cloned isolate of OpMNPV (Quant-Russell *et al.,* 1987) was used to infect *L. dispar* cells. The AcMNPV and *Spodoptera frugiperda* isolates used were described previously (Russell and Rohrmann, 1993).

DNA sequence analysis

Plasmid templates for sequencing were prepared using Qiagen columns (Qiagen Inc.) or by a protocol recommended by Applied Biosystems, Inc. that involved SDS–alkaline lysis followed by polyethylene glycol precipitation. Sequencing reactions were performed using the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Inc.) according to the manufacturer's protocol, with the exception that the reactions were performed in 5% DMSO and cycled to a higher denaturation temperature (97°). A Perkin–Elmer–Cetus Model 480 or Model TC1 thermal cycler was used. Reactions were electrophoresed and analyzed on an ABI Model 373A Automated DNA Sequencer. The nucleotide sequence and the predicted protein sequence were analyzed with the GCG suite of sequence analysis programs (Devereux *et al.,* 1984).

RNA isolation

For RNA isolation, approximately 9×10^6 *L. dispar* cells were infected with OpMNPV at an m.o.i. of 10, harvested at various times postinfection, mixed with 1 ml Trizol (Gibco BRL), and RNA was isolated according to the manufacturer's directions.

Northern blot analysis

In order to analyze the temporal expression of the ORF2 mRNA, RNA samples (20 μg) from the time course of OpMNPV-infected *L. dispar* cells were electrophoresed through a 1.25% agarose/6% formaldehyde gel in 20 m*M* MOPS buffer (AMRESCO) and transferred to a Gene Screen Plus (Dupont) membrane. The RNA was crosslinked to the membrane using a uv crosslinker (UV Stratalinker 1800, Stratagene, Inc.) according to the manufacturer's directions. The membrane was then baked for 2 hr at 80°. The Northern blot was prehybridized, hybridized, and washed as previously described (Ahrens and Rohrmann, 1995). In order to generate a cRNA transcript complementary to *orf2,* an 890-bp *Acc*I fragment from within *orf2* (Fig. 1) was cloned into pBS(−). This clone was linearized with *Hin*dIII and a labeled transcript was generated using T7 RNA polymerase and [^{32}P]UTP (Blissard *et al.,* 1989).

Primer extension and 3′ S1 analysis

To identify the ORF2 mRNA start site, an oligonucleotide complimentary to the mRNA of ORF2 was synthesized and used in primer extension analyses as previously described (Blissard *et al.,* 1989). The location of the primer is shown in Fig. 2. The primer was annealed to RNA at 50° and extended at 42°. Primer extension products were sized on a 6% polyacrylamide–7 *M* urea gel.

To locate the termination site of ORF2 mRNA, 3′ S1 mapping was carried out as described by Favaloro *et al.* (1980). A 1.4-kb *Acc*I/*Nru*I fragment (Fig. 1) was 3′ end-labeled with [α-^{32}P]dCTP and Klenow according to Sambrook *et al.* (1989), hybridized to 8 μg RNA from OpMNPV-infected *L. dispar* cells, and treated with S1 nuclease. S1-protected fragments were run on 8% polyacrylamide/7 *M* urea gels next to a ^{32}P-labeled 1-kb ladder (Gibco BRL) and a DNA sequencing ladder.

Construction of pMALcR1–ORF2 fusion and antibody production

The cloning strategy for constructing a maltose binding protein–ORF2 protein fusion (MBP–ORF2) involved removing a 2.5-kb *Sal*I fragment from pBB (Leisy *et al.,* 1986) and subcloning it into the *Sal*I site of pMALcR1. The recombinant plasmids were transformed into DH5α cells and screened for orientation. This resulted in an in-frame fusion beginning at amino acid 190 (Fig. 2) and extending to the end of the gene. Extracts from transformants containing the insert in the proper orientation were screened on Western blots using the antiserum to preoccluded virions previously described (Russell and Rohrmann, 1993). DNA from those clones producing a fusion protein recognized by the preoccluded virion antiserum was used to transform TOP10 cells (Invitrogen, Inc.). TOP10 transformants were grown to an $OD_{(595)}$ of 0.5–0.6, induced with IPTG at a final concentration of 0.3 m*M,* and incubated 2 hr at 37°. Protein extracts were generated as previously described (Russell and Rohrmann, 1993). The fusion protein was electrophoresed through 10% SDS–polyacrylamide gels and eluted as

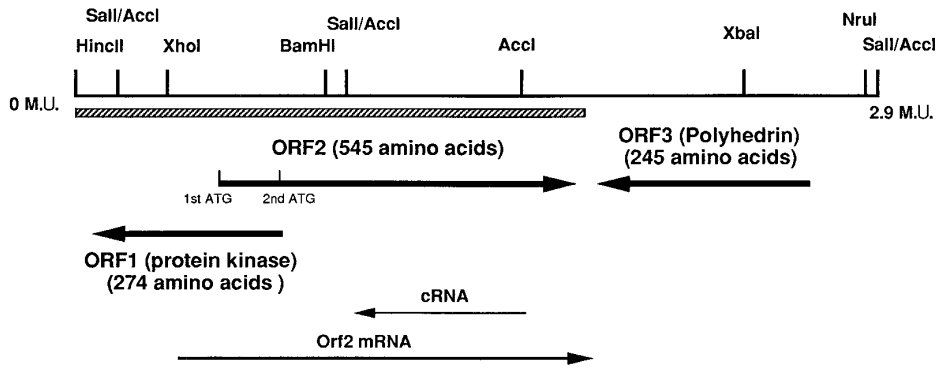

FIG. 1. Location of the *orf2* gene on the OpMNPV genome relative to the polyhedrin gene and the protein kinase homolog. Prominent restriction enzyme sites are indicated and the crosshatched box shows the location of the sequence presented in Fig. 2. The location of the cRNA used for Northern blot analysis and the 1.85-kb *orf2* transcript are also indicated.

described (Russell and Rohrmann, 1993). Protein concentrations were estimated by running aliquots of the purified fusion protein on a SDS–polyacrylamide gel against a standard curve of β-galactosidase. A New Zealand White rabbit was injected with 200 μg fusion protein emulsified in complete Freund's adjuvant. The rabbit was boosted with at least 250 μg fusion protein emulsified in incomplete Freund's adjuvant after 4 weeks and every 3 weeks thereafter, and immune serum was collected after the third boost. The antibody to the maltose binding protein was purchased from New England Biolabs, Inc.

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HincII
GTTGACGACCCCGACGTGTGCCTAAACGCACACGTCCCCACGCAGTTGGTGCTCAACTCGGCGGCGGCGGCGAACGACACCAATGACGCGCCGCCCGATCCGCCGCCGGCCCAATGCACC  120
                                                    SalI/AccI
ATTCAAATATAGTCGTGTCGGCTGGCCAAAAACGAATGTTTTGCAATCTGCTTGTAGTCGGTCGACCTGCACTCAAAGTTGAACCTGGTCAAGCAAAACACAAAGTCGCGCGCCATCAAA  240
      I  Y  D  H  R  S  A  L  F  S  H  K  A  I  Q  K  Y  D  T  S  R  C  E  F  N  F  R  T  L  C  F  V  F  D  R  A  M  L

TTGCGCACGTTTTTCAGCGCGGCGGGGTCGTTGTATTGCTGGCGTCGCCGCATGCTGGCCAGGTCCAGCACCTCGTCCGCGCTCCGCTCAAACGGATGCCGGCCCCCGGTTAGGAGTTTG  360
 N  R  V  N  K  L  A  A  P  D  N  Y  Q  Q  R  R  R  M  S  A  L  D  L  V  E  D  A  S  R  E  F  P  H  R  G  G  T  L  L  K
                                                                                  XhoI
TAGGCTAGCACGCCGACGGGCGTACCAGTCGAATGACCGCGCGTAGTTGTGGCGGCGAATTTTTTCCGGGCTAAAGTACTCGAGCGGTGCCGTCGTGCACCGGCGAGTGTTCGCGCTTGCAC  480
 Y  A  L  V  G  V  A  Y  W  D  F  S  R  A  Y  N  H  R  R  I  K  E  P  S  F  Y  E  L  T  G  D  H  V  P  S  H  E  R  K  C
                                  •5'->
AGGCCGTAGTCGCAAAGATACACGCGATCCCGCGCCCCAAAGTATAAGACGTTCTCCAGCTTGACGTCGTTGTGGATGTAGCCGGTGGCGTTGTGCAAGTCGTTCAACGCGTCGCACAAC  600
 L  G  Y  D  C  L  Y  V  R  D  R  A  G  F  Y  L  V  N  E  L  K  V  D  N  H  I  Y  G  T  A  N  H  L  D  N  L  A  D  C  L
                                                                                                    primer
TGCCGCACTATATTAACCACCAGCGCGTTGTCAAGCGCGCCCTGCGTTTGCAGCGTCTCAAACAGGTCCGGGCACGGCACGTAGTCCATCACAATTGCCCAAGCGGTGGGCGAGCTGTAG  720
 Q  R  V  I  N  V  V  L  A  N  D  L  A  G  Q  T  Q  L  T  E  F  L  D  P  C  P  V  Y  D  M  V  I  A  W  A  T  P  S  S  Y

CAAAAGTACATGTCCACAAAGCTGGGGTGGTCAGACATTAAATCGTGCACGTTGATTTCGTCGGCGCTAAAGCTGTGCGCGGCAATTGTTTTGCGCAGAAACAGCTTGCTGGTGGTGGGG  840
  ORF2-> M  S  T  K  L  G  W  S  D  I  K  S  C  T  L  I  S  S  A  L  K  L  C  A  A  I  V  L  R  R  N  S  L  L  V  V  G    37
 C  F  Y  M  D  V  F  S  P  H  D  S  M  L  D  H  V  N  I  E  D  A  S  F  S  H  A  A  I  T  K  R  L  F  L  K  S  T  T  P
                                                                                                ORF2 2nd ATG
CGGTGGTGCAGCACGTCCATTTTGCCAAATCTTCCGTTGACCACTTTGGGCGCGAGAACGGCGCAGTCGGCCACGAACCGGCTTAACGACTGCAGGGCCGCGTCCATGATGGAGCGACAG  960
R  W  C  S  T  S  I  L  P  N  L  P  L  T  T  L  G  A  R  T  A  Q  S  A  T  N  R  L  N  D  C  R  A  A  S  M  M  E  R  Q    77
 R  H  H  L  V  D  M  K  G  F  R  G  N  V  V  K  P  A  L  V  A  C  D  A  V  F  R  S  L  S  Q  L  A  A  D  M <-ORF1

TATCAATCCGTTCGGTCTTATCTAATCAACAACCAGCACAACGCCATTGCTGCGGGCCCGTTCTTGCAGCGCGTGGCGGGGCCCGAGGCGCACAGCGTTGGGCGCAACGTTGGCGACCGC 1080
Y  Q  S  V  R  S  Y  L  I  N  N  Q  H  N  A  I  A  A  G  P  F  L  Q  R  V  A  G  P  E  A  H  S  V  G  R  N  V  G  D  R    117

GCGGTCACGCTCAACAGGCAAGCGGTTTTGGATCTACTAAAACTGGCCGAAGACATTTACGCGGACACGGCGTACATGCAAGCGGACCAGCCAGAGCGTTCGTCGCGGCACTTTGCCACG 1200
A  V  T  L  N  R  Q  A  V  L  D  L  L  K  L  A  E  D  I  Y  A  D  T  A  Y  M  Q  A  D  Q  P  E  R  S  S  R  H  F  A  T    157
                                BamHI                                                                 SalI/AccI
CTGAACCGCATGCGCCTGCTCCTGATCGGCGTGCAGGATCCCGACGCCCGGCGCAATTTAAACAGTGTTTGGCGCGCATTGAAGCGCTGTTGCGCGTCGACGTGGTGAACGATGCGGAG 1320
L  N  R  M  R  L  L  L  I  G  V  Q  D  P  D  A  R  R  N  L  N  S  V  L  A  R  I  E  A  L  L  R  V  D  V  V  N  D  A  E    197
                                                                                                    RI
GTTAACGTGCTGAGCGGCGATTTCTACGAGGAGTATTCCAAATACATTTCTTACCAACAAACCTTTGCGCAAACGCCACCGCTTCTGCTTCGCAACAAACACAAACCTCTCTTCCGCGGC 1440
V  N  V  L  S  G  D  F  Y  E  E  Y  S  K  Y  I  S  Y  Q  Q  T  F  A  Q  T  P  P  L  L  L  R  N  K  H  K  P  L  F  R  G    237
          RI                                RI
CACAAACCTCTCTTCCGCGGCAAACGCAGGCCTCTCTTCCGCAACAAACGCCCTTTGATCAGCCTGAAATGGTCTCGCCGCCGTCCTTTGTACACACAACGCCGCTATTTTGGGCGAAA 1560
H  K  P  L  F  R  G  K  R  R  P  L  F  R  N  K  R  P  L  I  S  L  K  W  S  R  R  R  P  L  Y  T  Q  R  P  L  F  W  A  K    277

ACCACGCAACCGCCGGCGACCGATACATTTCTCGGCCCTCTGACGAGTTTGTTTATGTGCCCGGCAAAGAACGCGCTGTGCCCGACACGCGATTTAAGCCTCCCGTGCCTCCTAAACCC 1680
T  T  Q  P  P  A  T  D  T  F  S  R  P  S  D  E  F  V  Y  V  P  G  K  E  R  A  V  P  D  T  R  F  K  P  P  V  P  P  K  P    317
                                                                                  RII
GAGCATTTAAAATCGCGGCCCTCTTCTGTGGCCACAAATGCTGCGGGCGCGACACCGGTCGCGCCGCCGCCGCCATTTCCGTCGGCGGATGTGACCACGAGCATGCCGCCGCCGCCGCG 1800
E  H  L  K  S  R  P  S  S  V  A  T  N  A  A  G  A  T  P  V  A  P  P  P  P  F  P  S  A  D  V  T  T  S  M  P  P  P  P  P    357
                  RII                                   RII                                   RII
CCATTTCCGTCGGCGGATGTGACCACGAGCATGCCGCCGCCGCCGCCAATGGTAGATTTGGCCACGAGCATGCCGCCGCCGCCGCCGCCGCCGCCAATGGTAGATTTGGCCACGAGC 1920
P  F  P  S  A  D  V  T  T  S  M  P  P  P  P  P  P  M  V  D  L  A  T  S  M  P  P  P  P  P  P  P  P  P  M  V  D  L  A  T  S    397

ATGCCGCCGCCAATTAACAACGCCATTAATAATTTATTAATAGACGCGATGGTCGCAGAGACAAACAAAAACGCGGGCGACAATCGCAGCGCGTTGCTTGATCAAATCAAACAAGGCAAA 2040
M  P  P  P  I  N  N  A  I  N  N  L  L  I  D  A  M  V  A  E  T  N  K  N  A  G  D  N  R  S  A  L  L  D  Q  I  K  Q  G  K    437

ACGCTTAAAAAAACCCAACCCGCAGACGGTGCTCCGGCAACCGACCCGCGCAGTACGCTGCTCAGCGAAATTAGACAAGGCAAAACTCTTAAAAAGCTGCGTAAAATCGAAGACCAAAGT 2160
T  L  K  K  T  Q  P  A  D  G  A  P  A  T  D  P  R  S  T  L  L  S  E  I  R  Q  G  K  T  L  K  K  L  R  K  I  E  D  Q  S    477
                AccI
TCAACGCAGACTTTGCTCAAAGACGTAGACACGACCGACAAAACAAAAACGATTTTGAAAAACTTTGTAACCAACATAGACAGGATTTCAAAGCAAGAACAAGAGGAAAAGGACCGGCTC 2280
S  T  Q  T  L  L  K  D  V  D  T  T  D  K  T  K  T  I  L  K  N  F  V  T  N  I  D  R  I  S  K  Q  E  Q  E  E  K  D  R  L    517
                                                                                  ••3'
GATACAATTACAAAACGCCGGCCCGCCGTGGAGCATACGGACGGCAACAGCACGGGCAACAACTCAGATGATTGGAGAGACGACTAGTAAAATAATTTGCAAAATTTAATTGTTTGTATT 2400
D  T  I  T  K  R  R  P  A  V  E  H  T  D  G  N  S  T  G  N  N  S  D  D  W  R  D  D  *                                    545
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FIG. 2. Nucleotide sequence of the OpMNPV *orf2* gene region. The location of the 25-mer oligonucleotide primer used for primer extension analysis of the *orf2* mRNA initiation site is doubly underlined. The location of late promoter/mRNA start and termination sites, along with major restriction sites, are indicated. Numbers to the right of the sequences indicate the positions of nucleotides and ORF2 amino acids. The two sets of repeated amino acids sequences are underlined and labeled RI and RII, respectively.

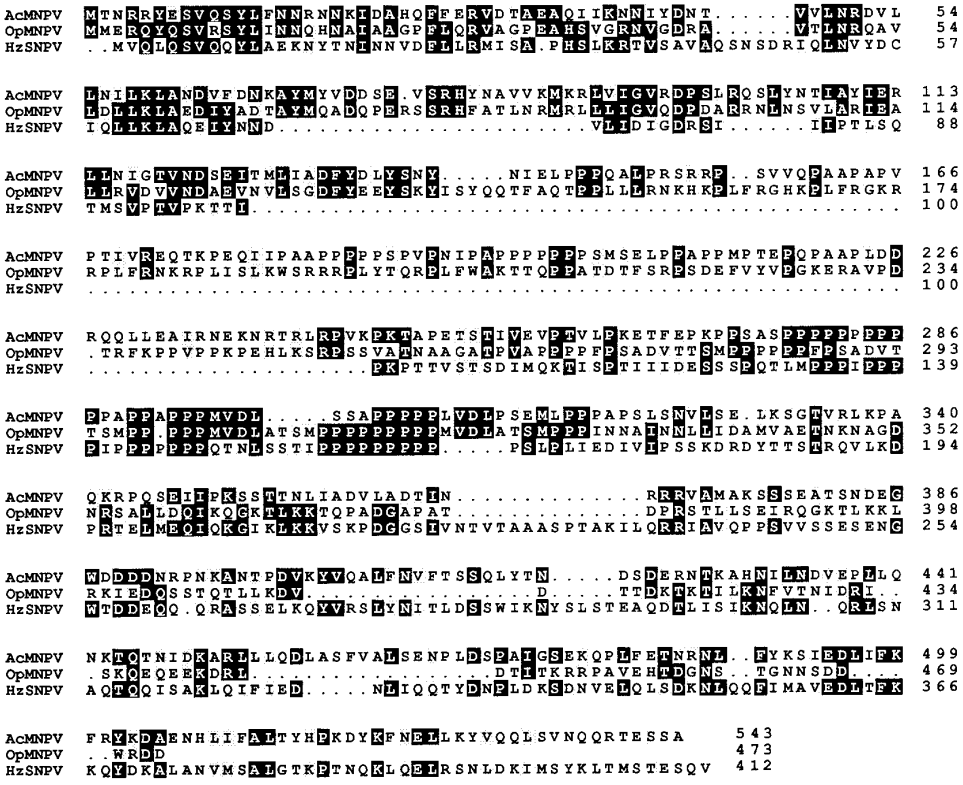

FIG. 3. Comparison of the predicted amino acid sequence of ORF2 homologs from AcMNPV (ac), OpMNPV (op), and HzSNPV (hz). Identical amino acids are indicated by white letters within black boxes; similar amino acids are shown within speckled boxes. Dots (.) indicate gaps. Amino acid number is indicated on the left.

Western blot analysis of OpMNPV- and AcMNPV-infected cells

Monolayers of *L. dispar* were infected with OpMNPV at an m.o.i. of 10 and harvested at various times postinfection. Cellular extracts were prepared as previously described (Quant-Russell *et al.,* 1987). Production of the time course of AcMNPV-infected *S. frugiperda* was described by Rasmussen and Rohrmann (1994). Samples were electrophoresed through 9% SDS/polyacrylamide gels (Laemmli, 1970), electroblotted onto nitrocellulose (Micron Separations Inc.) for 2 hr at 185 mA, and Western blot analyses were carried out as previously described (Quant-Russell *et al.,* 1987). Antiserum to the maltose binding protein–ORF2 fusion was used at a dilution of 1:5000 to 1:12,500. Antiserum to MBP (New England Biolabs) was used at dilutions of 1:10,000. Antisera were preadsorbed with 5 μg/ml mouse brain powder (Sigma) for 2 hr at room temperature before using.

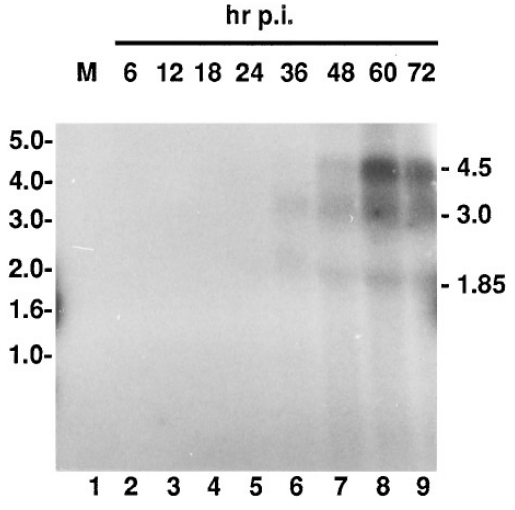

FIG. 4. Northern blot analysis of the transcript from the orf2 region. A ^{32}P-labeled cRNA probe complementary to orf2 was hybridized to RNA blotted onto Gene Screen Plus. The sizes (kb) of selected markers are indicated at the left. The sizes (kb) of major transcripts are shown at the right. The numbers at the top indicate the hours postinfection (hr p.i.) that the RNAs were extracted. M is mock-infected.

Immunoelectron microscopy

Samples for immunoelectron microscopy of OpMNPV-infected *L. dispar* cells were prepared and immunoelectron microscopy was carried out as described previously (Russell and Rohrmann, 1990). Preimmune and MBP–ORF2 antisera were used at dilutions of 1:1000. MBP antiserum was used at a concentration of 1:5000 which was calculated based on the relative intensity of its reaction on Western blots. Goat anti-rabbit IgG F(ab′)2 conjugated with 10-nm gold particles was used at a dilution of 1:75. Samples were observed with a Philips EM 300 electron microscope.

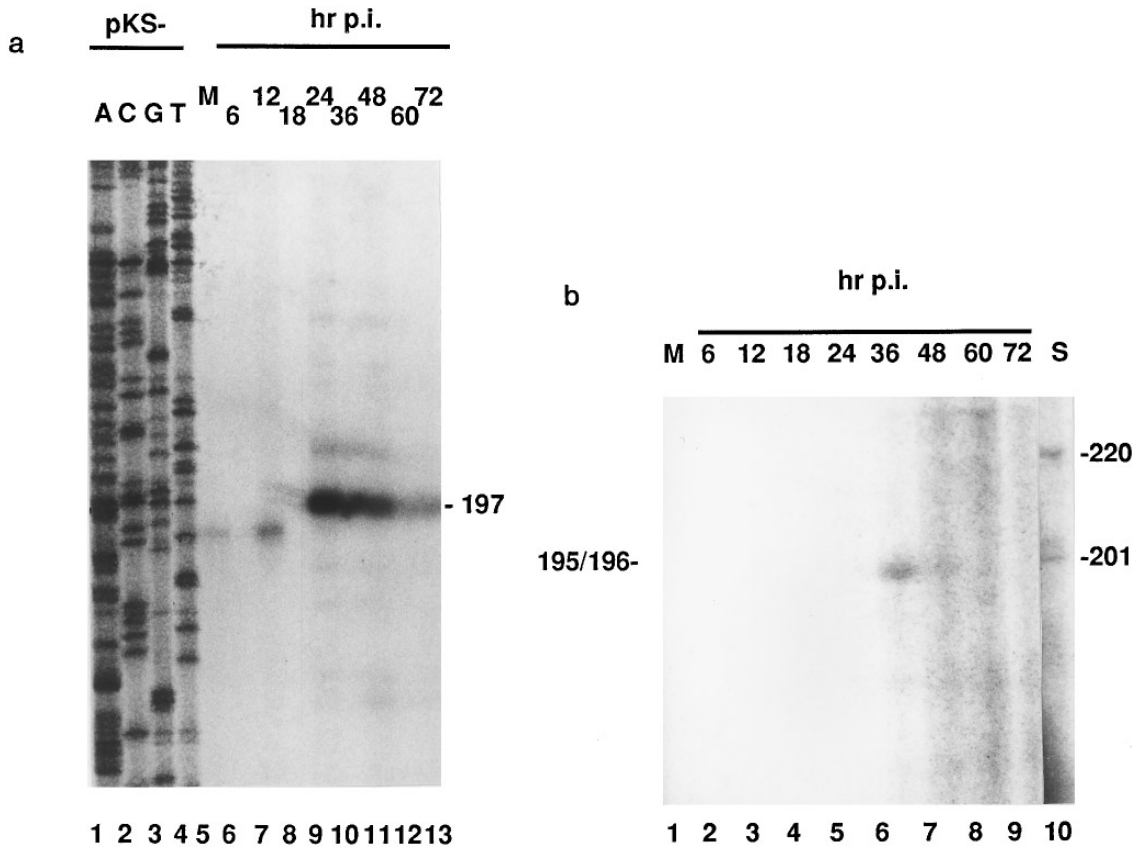

FIG. 5. 5′ and 3′ transcriptional mapping of the OpMNPV orf2 transcript. (a) Primer extension analysis of the orf2 transcript from different hours postinfection. The sizes (nt) of the primer extension products are indicated on the right and were determined by alignment with a pBKS-sequence ladder. (b) 3′ mapping of the orf2 transcripts by S1 nuclease analysis. The size of the product (indicated on the left margin) was determined by comparison to ^{32}P-labeled 1-kb ladder (BRL) (right lane S) and confirmed with a DNA sequencing ladder that was electrophoresed on the margins of the gel.

RESULTS

Location and nucleotide sequence of the OpMNPV homolog of AcMNPV ORF9

Nucleotide sequence analysis of the region downstream of the OpMNPV polyhedrin gene (Fig. 1) led to the identification of a large open reading frame (*orf2*) that is in the opposite orientation of polyhedrin and encodes a predicted protein of 545 amino acids (Fig. 2) with a molecular weight of 60.5 kDa. Although the predicted size is close to that observed on Western blots (69 kDa — see below), the first ATG, at nt 730 (Fig. 2), is in poor translational context (Kozak, 1986) with T's at both the −3 and +4 positions. In addition, alignment with the AcMNPV ORF9 (Fig. 3) suggested that the OpMNPV ORF2 started at a second in-frame ATG (nt 946), since the AcMNPV ORF9 showed no homology to the region upstream of the OpMNPV ORF2 second ATG. Furthermore, the AcMNPV ORF10 (protein kinase homolog) shows a high degree of homology to an overlapping ORF starting at nt 947 and in the opposite orientation of ORF2. If the second ATG is used, the OpMNPV ORF2 would encode a protein predicted to encompass 473 amino acids with a molecular weight of 53 kDa, which is significantly smaller than the 69-kDa size observed on Western blots (see below). However, the AcMNPV ORF9 protein appears to migrate at a significantly higher mass than would be predicted from the sequence. This has been attributed to aberrant migration on SDS–PAGE gels caused by the high content of prolines present in the protein (Pham and Sivasubramanian, 1993; Vialard and Richardson, 1993) and allows for the possibility that ORF2 starts at the downstream ATG.

AcMNPV ORF9 is not well-conserved with its homologs from OpMNPV and HzSNPV. The AcMNPV gene showed a predicted amino acid sequence identity of 29% with the two other ORFs and the OpMNPV and HzSNPV ORFs showed only 19% sequence identity to each other. However, there were two areas of higher sequence conservation in the predicted sequence. AcMNPV and OpMNPV had 45% identity in the first 150 amino acids (Fig. 3). The second region of similarity was a proline-rich region near the middle of the molecule. This region was most extensive in AcMNPV and least in HzSNPV. In OpMNPV, a sequence of 66 amino acids (amino acids 264–329) contained 45% proline residues. The 123 amino

acids (amino acids 141–263 in OpMNPV) directly upstream of this proline-rich region in OpMNPV shows little homology with AcMNPV and is entirely absent in HzSNPV.

A leucine zipper present in the AcMNPV ORF9 (Ayres *et al.,* 1994) (beginning at amino acid 316, Fig. 3) is not conserved in the OpMNPV and HzSNPV homologs. A series of amino acid repeats are present in OpMNPV ORF2. These include three repeats of the sequence related to PLFRG and four larger repeats related to (PPPPFPSADVTTSMPP) (Fig. 2). The larger repeats appear to have been derived by duplication of GCC repeat sequences as discussed previously (Chen *et al.,* 1988). Whereas the PLFRG repeat is not conserved between the other ORF2 homologs, the larger proline-rich repeat does show some homology with similar proline-rich regions of the other two sequences (Fig. 3).

Transcriptional mapping of ORF2

Northern blot, primer extension, and 3′ and 5′ S1 nuclease protection assays were carried out to study the regulation of transcription of the ORF2 gene. A labeled cRNA complementary to an 890-bp *Acc*I fragment from ORF2 (Figs. 1 and 2; nt 1298–2188) was synthesized and then hybridized to a Northern blot of total RNA isolated from OpMNPV-infected *L. dispar* cells at various times postinfection. Three transcripts were identified (Fig. 4). A band of approximately 1.85 kb was detected at 24 hr p.i. Additional bands of approximately 3.0 and 4.5 kb were apparent at 36 hr p.i. All three transcripts were present in all subsequent time points, but were most abundant at 60 hr p.i., at which time the 4.5-kb transcript was the predominant species.

The transcriptional start site was determined by primer extension analysis of total RNA from infected *L. dispar* cells using a DNA oligonucleotide complementary to sequences near the 5′ end of ORF2 (Fig. 2). The exact location of the transcriptional start was determined by comparing the size of the primer extension product with a pBluescript KS$^-$ sequencing ladder (Fig. 5a). Primer extension from within ORF2 resulted in a major product of 197 nt (Fig. 5a) corresponding to the T of the ATAAG late promoter upstream of the first ATG of ORF2 (Fig. 2). The signal was first detected and was most intense at 24 hr p.i., and by 60 hr p.i. the signal had decreased considerably. An additional band was observed just downstream of the late promoter at 12 hr p.i., but the same signal was also observed with the mock-infected (0 hr) time point.

To map the 3′ end of the ORF2 transcript, a 1.4-kb *Acc*I (Figs. 1 and 2; nt 2186) to *Nru*I (Fig. 1) fragment that contained the 3′ end of ORF2 as well as the polyhedrin gene was 3′ end-labeled and hybridized to total RNA from OpMNPV-infected *L. dispar* cells, then treated with S1 nuclease. The reactions were electrophoresed on an

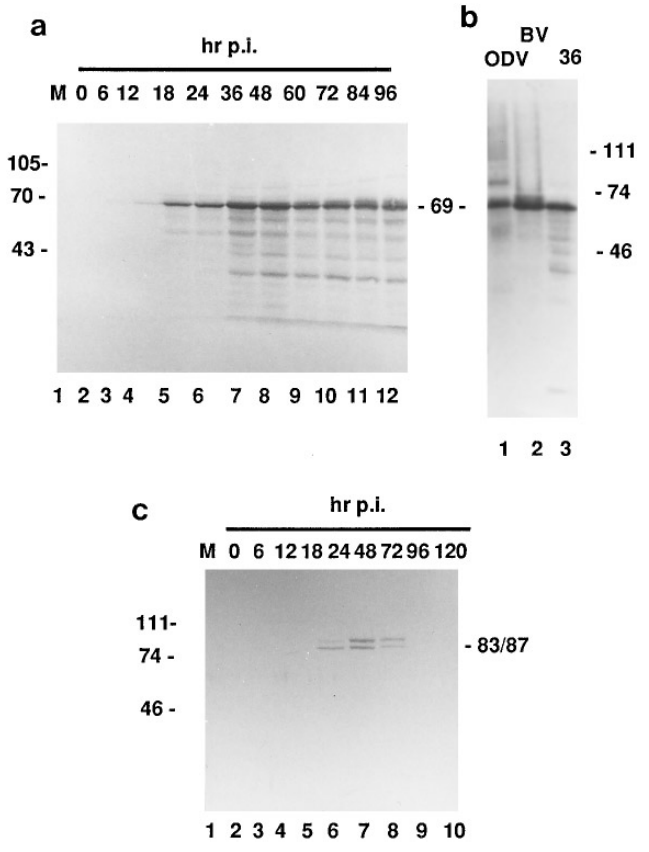

FIG. 6. Western blot analysis of ORF2. (a) Expression of ORF2 in OpMNPV-infected *L. dispar* cells. The numbers on the top of each lane indicate hours postinfection. Mock-infected cells are indicated by the letter M. (b) Western blot analysis of ORF2 in purified OpMNPV budded and occluded viral phenotypes. Lanes include: ODV, BV, and 36 hr p.i. cell extract. (c) Western blot analysis of extracts of AcMNPV-infected *S. frugiperda* cells using the OpMNPV MBP-ORF2 antiserum. Sizes (kDa) of the protein standards are indicated on the left (a,c) and right (b); the size of ORF2 is indicated on the right (a, c) and left (b).

8% polyacrylamide/7 *M* urea gel and protected bands of 195 and 196 nt were present at 24, 36, and 48 hr p.i. (Fig. 5b). These bands mapped to two A's (Fig. 2; nt 2369, 2370) just two and three bases downstream of the termination codon for ORF2. The data from the 3′ and 5′ mapping predicted a transcript of 1.85 kb, which is similar in size to the smaller transcript identified on Northern blots.

Temporal expression of the ORF2 protein

In order to study the expression of the ORF2 gene product, an antibody was produced to a MBP–ORF2 fusion protein. Time courses of OpMNPV-infected *L. dispar* and AcMNPV-infected *S. frugiperda* cells were analyzed by Western blot using the MBP–ORF2 antiserum. A major immunoreactive band of approximately 69 kDa was first observed at 6 hr p.i. in the OpMNPV time course (Fig. 6a). This band stained most intensely at 36 and 48 hr p.i., at which time a slightly larger band was also observed. These two bands were present in

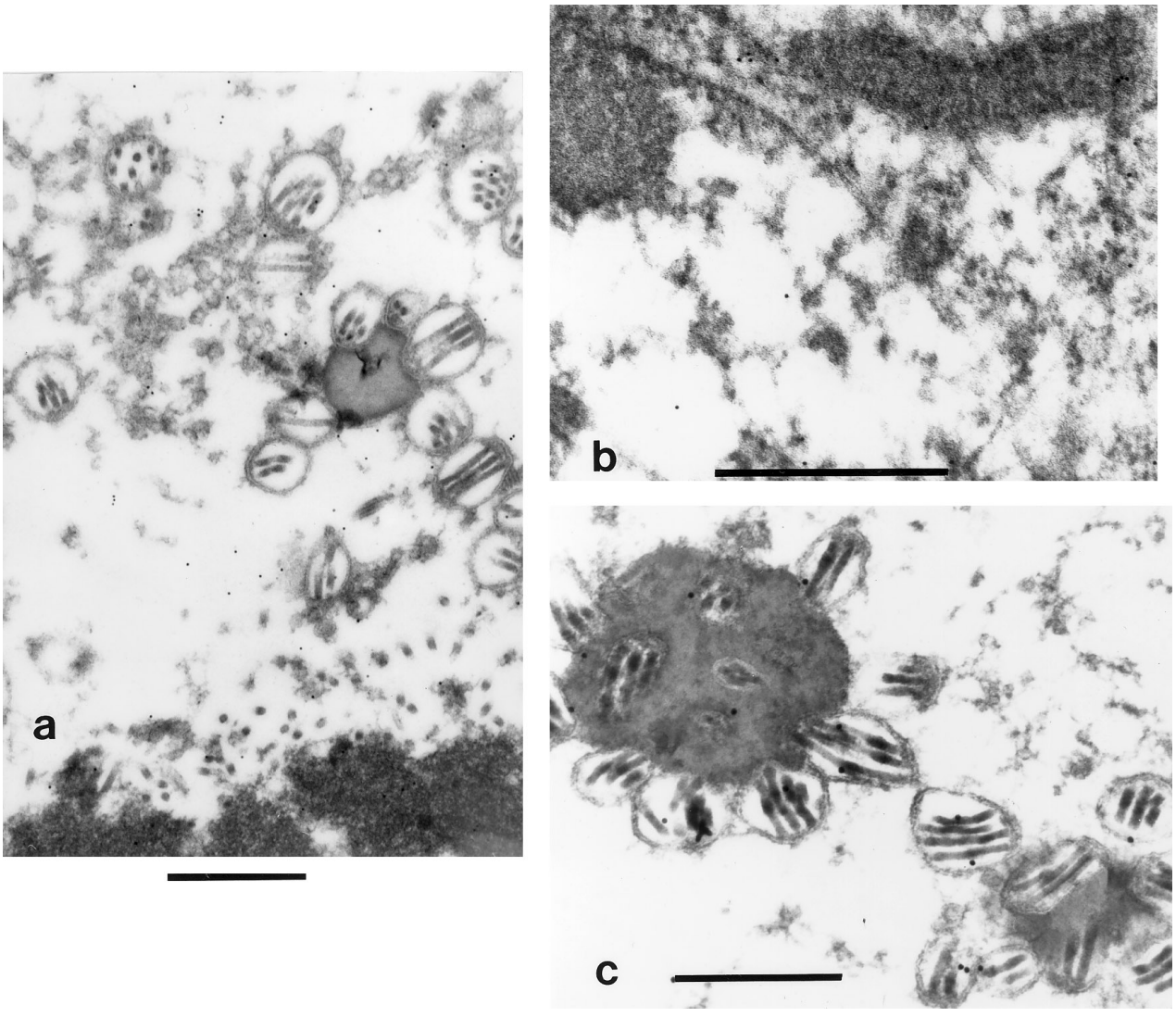

FIG. 7. Immunogold staining controls. (a) Cells infected at 48 hr p.i. and incubated with preimmune serum at a dilution of 1:1000. (b) Uninfected cells stained with MBP-ORF2 antibody. The MBP-ORF2 antibody was used at a dilution of 1:1000. (c) Cells infected at 48 hr p.i. and stained with maltose binding protein antibody. The primary antibody was diluted 1:5000. (a–c) The gold-conjugated secondary antibody was used at 1:75. The bars indicate 0.5 μm.

all later time points. A number of smaller immunoreactive bands were also observed, especially at 36 hr p.i. and later, which may be due to protein breakdown. To determine if the protein recognized by the antiserum was associated with both viral phenotypes, Western blot analysis was done on OpMNPV PDV and BV and compared to an infected cell extract from 36 hr pi. The antiserum reacted with a band in both phenotypes that was similar in size (69 kDa) to the band observed in infected cell extracts (Fig. 6b). However, BV may have more of the higher molecular weight species. The antiserum was also tested against the AcMNPV time course where it recognized a doublet of 83 and 87 kDa (Fig. 6c), which is similar in size to that previously described (Pham and Sivasubramanian, 1993; Vialard and Richardson, 1993). The doublet was first observed at 24 hr p.i., was most intense at 48 hr p.i., and was no longer observed at 96 and 120 hr p.i.

Localization of the ORF2 gene product

To determine the localization of the OpMNPV ORF2 within infected *L. dispar* cells, immunoelectron microscopy was carried out. Preimmune serum showed low background staining of OpMNPV-infected cells at 48 hr p.i. (Fig. 7a) as did antiserum to the MBP–ORF2 fusion protein when incubated with uninfected *L. dispar* cells (Fig. 7b). OpMNPV-infected *L. dispar* cells at 48 hr p.i. incubated with a monoclonal antibody against MPB showed little background staining (Fig. 7c). The antiserum to MBP–ORF2 was incubated with sections of infected cells from a series of timepoints postinfection and

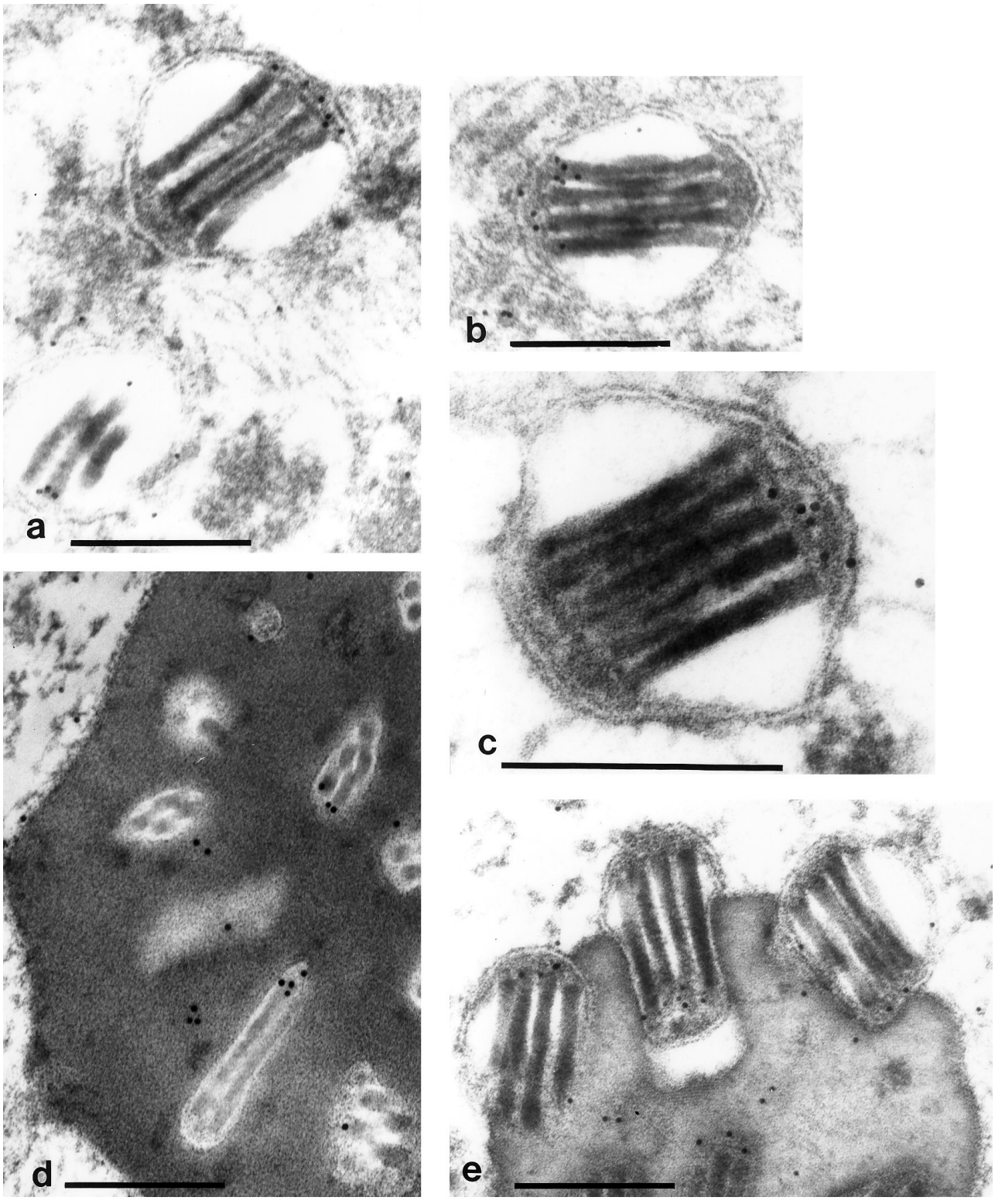

FIG. 8. Immunogold staining of enveloped virions in nuclei with MBP-ORF2 antiserum. (a and b) 96 hr p.i.; (c) 48 hr p.i.; (d) 72 hr p.i.; (e) 84 hr p.i. Primary antibody, 1:1000; secondary antibody, 1:75. The bars indicate 0.25 μm.

appeared to associate with the ends of the virus particles in both preoccluded and occluded stages (Figs. 8a–8e). Longitudinal sections of enveloped virions that showed both end structures were examined to determine whether only one end or both ends of the virions are recognized by the antiserum. Our results indicated that the antiserum bound to only one end of the virion (Figs. 8a–8e).

Late in the course of infection, aggregates of virions

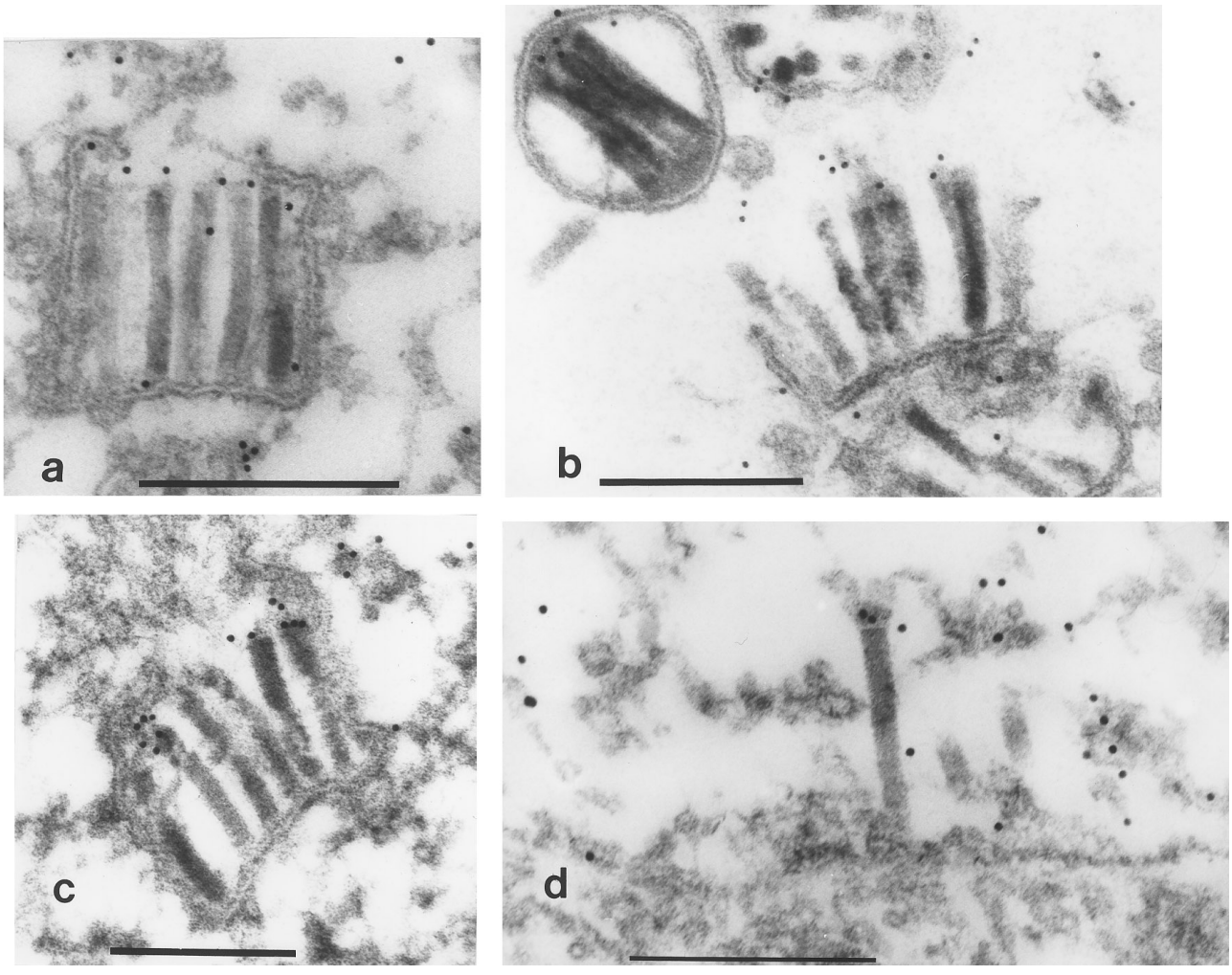

FIG. 9. Immunogold staining of virions. (a–c) Virions in the process of envelopment at 72 hr p.i. (a), 48 hr p.i. (b), and 84 hr p.i. (c). (d) Virion at the nuclear membrane, 48 hr p.i. MBP-ORF2 antibody, 1:1000; gold-conjugated secondary antibody, 1:75. The bar indicates 0.25 μm.

are enveloped prior to being occluded in polyhedra. These "precursor" structures, composed of virions aligned along a double membrane/envelope, with one end of the virion associated with the membrane and the other end unattached, are often observed. Since the ORF2 gene product appeared to be specific for a particular end of the virion, we examined a number of these intermediate structures to determine if the virions were found in a particular orientation when associating with the envelope. Immunogold staining of these structures was not intense, but the antiserum appeared to recognize the end of the virion that was not attached to the membrane (Figs. 9a–9c). We also looked for virions budding through the nuclear membrane to determine if a particular orientation of the virion was necessary for this process. Although immunogold staining of single virions was limited, the end of the virion lacking ORF2 appeared to associate with the nuclear membrane in a number of virions (Fig. 9d).

Immunogold staining of the virogenic stroma was also examined with the MBP–ORF2 antiserum (Fig. 10). Gold particles were most apparent in regions around the edges of the electron-dense region of the stroma. Virions appeared to be oriented toward the virogenic stroma and ORF2 appeared to be associated with the end directed away from the stroma in a number of the virions (Fig. 10, see arrows).

DISCUSSION

In the nucleus of baculovirus-infected cells a virogenic stroma forms which is composed of a homogeneous matte-like structure that is highly sensitive to RNase and which has discrete compartments containing DNA (Young *et al.,* 1993). It contains intrastromal spaces and has a convoluted periphery and has been implicated as being the site of DNA synthesis and nucleocapsid assembly. Electron micrographic studies (Fraser, 1986) suggest that the assembly of capsid sheaths occurs within the intrastromal spaces of the virogenic stroma.

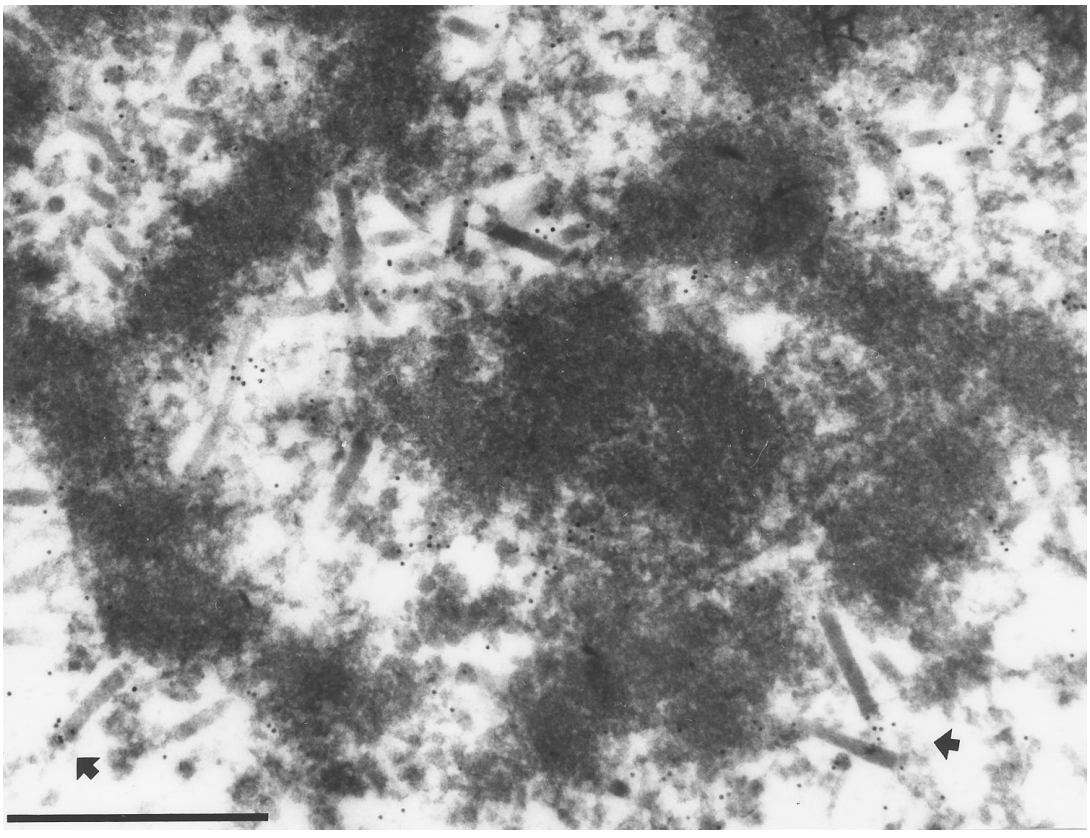

FIG. 10. Immunogold staining of virogenic stroma. Virogenic stroma at 48 hr p.i. Arrows indicate nucleocapsids with staining of the basal region. MBP-ORF2 antibody, 1:1000; gold-conjugated secondary antibody, 1:75. The bar indicates 0.5 μm.

The capsids are polar in structure with an apical cap structure at one end and a basal structure at the other. The empty capsids align with the apical end oriented toward the stroma and the DNA–protein (nucleoprotein complex) is thought to be transferred from the stroma into the capsid. A cap-like structure is often observed at the apex of partially filled capsids and may be involved in the packaging of the genomic DNA into the capsid (Fraser, 1986). The apical end structure also appears to associate with membranous vesicles in the nucleus during envelopment and the inner surface of the plasma membrane during budding (Fraser, 1986).

It was initially suggested that the AcMNPV ORF9 protein was localized to the virion envelope or intermediate layer of both ODV and BV (Pham *et al.,* 1993). However, evidence was subsequently presented suggesting that the ORF9 protein is localized to at least one end of the capsid (Vialard and Richardson, 1993). Our electron micrographs suggest that the OpMNPV ORF2 protein is associated with only the basal end of the capsid. The capsid end associated with ORF2 is directed away from the apparent interaction with the virogenic stroma and similarly is oriented away from the end associated with the initial interaction with membranes during the process of envelopment in the nucleus. In addition, we observed that the end associated with the ORF2 protein is oriented away from the nuclear membrane in virions that appear to be preparing to migrate from the nucleus to the cytoplasm in transit to the plasma membrane. Although we looked for virions budding through the plasma membrane, we were unable to find satisfactory examples. However, the ORF2 protein is probably not directly involved in this budding process as the basal capsid region has previously been shown to be directed away from the plasma membrane during this process (Fraser, 1986).

The data presented in this report suggest that the ORF2 protein may be associated with the basal end of the capsid and may be a component of a specific end structure of the capsid. The results suggest that specific proteins may be located in the apical cap end of the capsid which would facilitate the association with the virogenic stroma and insertion of nucleoprotein into the capsid sheath, the initial interactions with the developing ODV envelope, and direct interaction with the nuclear membrane. To date no proteins specific to the apical cap end of capsids has been reported.

Although ORF2 is associated with a specific viral structure, it is poorly conserved. However, the apparent cross-reactivity of the antibodies directed against the OpMNPV protein with the AcMNPV protein suggest that in spite of the sequence divergence, there is a certain degree of structural conservation. This structural conservation is

likely to be present even in the poorly conserved C-terminal region, because the OpMNPV ORF2 antibody that appeared to crossreact with the AcMNPV homolog was generated from a gene fusion lacking most of the conserved N-terminal region.

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REFERENCES

Ahrens, C. H., and Rohrmann, G. F. (1995). Identification of essential trans-acting regions required for DNA replication of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus: *lef-1* is an essential replication gene. *Virology* 207, 417–428.

Ayres, M. D., Howard, S. C., Kuzio, J., Lopez-Ferber, M., and Possee, R. D. (1994). The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* 202, 586–605.

Blissard, G. W., Quant-Russell, R. L., Rohrmann, G. F., and Beaudreau, G. S. (1989). Nucleotide sequence, transcriptional mapping, and temporal expression of the gene encoding p39, a major structural protein of the multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata*. *Virology* 168, 354–362.

Chen, D. D., Nesson, M. H., Rohrmann, G. F., and Beaudreau, G. S. (1988). The genome of the multicapsid baculovirus of *Orgyia pseudotsugata:* Restriction map and analysis of two sets of GC-rich repeated sequences. *J. Gen. Virol.* 69, 1375–1381.

Cowan, P., Bulach, D., Goodge, K., Robertson, A., and Tribe, D. E. (1994). Nucleotide sequence of the polyhedrin gene region of *Helicoverpa zea* single nucleocapsid nuclear polyhedrosis virus: placement of the virus in lepidopteran nuclear polyhedrosis virus group II. *J. Gen. Virol.* 75, 3211–3218.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12, 387–395.

Favaloro, J., Treisman, R., and Kamen, R. (1980). Transcriptional maps of polyoma virus-specific RNA: analysis by two dimensional nuclease S1 gel mapping. *Methods Enzymol.* 65, 718–749.

Fraser, M. J. (1986). Ultrastructural observations of virion maturation in *Autographa californica* nuclear polyhedrosis virus infected *Spodoptera frugiperda* cell cultures. *J. Ultrastruct. Mol. Struct. Res.* 95, 189–195.

Granados, R. R., and Lawler, K. A. (1981). In vivo pathway of *Autographa californica* baculovirus invasion and infection. *Virology* 108, 297–308.

Kitts, P. A., and Possee, R. D. (1993). A method for producing recombinant baculovirus expression vectors at high frequency. *BioTechniques* 14, 810–816.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44, 283–292.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

Leisy, D., Rohrmann, G., and Beaudreau, G. (1986). The nucleotide sequence of the polyhedrin gene region from the multicapsid baculovirus of *Orgyia pseudotsugata. Virology* 153, 280–288.

Pham, D. Q.-D., Hice, R. H., Sivasubramanian, N., and Federici, B. A. (1993). The 1629-bp open reading frame of the *Autographa californica* multinucleocapsid virus encodes a virion structural protein. *Gene* 137, 275–280.

Pham, D. Q.-D., and Sivasubramanian, N. (1992). In vivo transcriptional analysis of three baculovirus genes: evidence of homology between viral and host transcripts. *Virology* 190, 288–297.

Pham, D. Q.-D., and Sivasubramanian, N. (1993). Sequence and in vitro translational analysis of a 1629-nucleotide ORF in *Autographa californica* nuclear polyhedrosis virus strain E2. *Gene* 122, 345–348.

Possee, R. D., Sun, T.-P., Howard, S. C., Ayres, M. D., Hill-Perkins, M., and Gearing, K. L. (1991). Nucleotide sequence of the *Autographa californica* nuclear polyhedrosis 9.4 kbp EcoRI-I and -R (polyhedrin gene) region. *Virology* 185, 229–241.

Quant-Russell, R. L., Pearson, M. N., Rohrmann, G. F., and Beaudreau, G. S. (1987). Characterization of baculovirus p10 synthesis using monoclonal antibodies. *Virology* 160, 9–19.

Rasmussen, C., and Rohrmann, G. F. (1994). Characterization of the *Spodoptera frugiperda* TATA-binding protein: Nucleotide sequence and response to baculovirus infection. *Insect Biochem. Mol. Biol.* 7, 699–708.

Russell, R. L. Q., and Rohrmann, G. F. (1990). A baculovirus polyhedron envelope protein: Immunogold localization in infected cells and mature polyhedra. *Virology* 174, 177–184.

Russell, R. L. Q., and Rohrmann, G. F. (1993). A 25 kilodalton protein is associated with the envelopes of occluded baculovirus virions. *Virology* 195, 532–540.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Vialard, J. E., and Richardson, C. D. (1993). The 1,629-nucleotide open reading frame located downstream of the *Autographa californica* nuclear polyhedrosis virus polyhedrin gene encodes a nucleocapsid-associated phosphoprotein. *J. Virol.* 67, 5859–5866.

Young, J. C., MacKinnon, E. A., and Faulkner, P. (1993). The architecture of the virogenic stroma in isolated nuclei of *Spodoptera frugiperda* cells in vitro infected by *Autographa californica* nuclear polyhedrosis virus. *J. Struct. Biol.* 110, 141–153.